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New host crab *Macrophthalmus* (*Macrophthalmus*) *convexus* Stimpson, 1858 of the endangered pedunculate barnacle *Octolasmis unguisiformis* Kobayashi & Kato, 2003 (Lepadomorpha: Poecilasmatidae) in Amami Oshima Island, Japan

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Abstract. — The pedunculate barnacle *Octolasmis unguisiformis* is a rare epibiotic barnacle attached to a macrophthalmid crab, *Macrophthalmus* (*Macrophthalmus*) *milloti* Crosnier, 1965, on tidal flats in the Ryukyu Archipelago. It has a rare sexual system called androdioecy, a coexistence of hermaphrodites and males in the population. In June 2020, we found two hermaphrodites and one dwarf male of *O. unguisiformis* attached to a female *M.* (*M.*) *convexus* at Kise Bay, Amami Oshima Island. Here we describe the symbiotic conditions between the pedunculate barnacle and the new host crab species.

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Key words: *epibiont*, *macrophthalmid*, *octolasmid*, *symbiosis*

■ Introduction

The barnacles of the genus *Octolasmis* (Lepadomorpha: Poecilasmatidae) are epibionts, living on corals, cnidarian, crabs, echinoderms, horseshoe crabs, and sea snakes (Jeffries & Voris, 1996, 2005). The host range of each species tends to be broad; for instance, *Octolasmis warwickii* lives on portunid crabs and horseshoe crabs (Jeffries *et al*., 1989; Li *et al*., 2015), and *O. lowei* has been recorded from six crab species (Negreiros-Fransozo *et al*., 1995). In contrast, *O. unguisiformis* Kobayashi & Kato, 2003 (Lepadomorpha: Poecilasmatidae) is a unique ectosymbiotic barnacle observed solely on a macrophthalmid crab, *Macrophthalmus milloti* Crosnier, 1965. This barnacle has only been recorded from waterlogged tidal flats of Amami Oshima, Kakeroma, Okinawajima, and Iriomote Islands (Kobayashi & Kato, 2003; Kato, 2012, Sawada *et al*., 2015). Due to such limited habitat and distribution, *O. unguisiformis* has been categorized as a threatened species inhabiting tidal flats in Japan (Kato, 2012). Interestingly, the appearance of this barnacle resembles the chela of the host crab in shape, size, and attachment location (Kobayashi & Kato, 2003). It has a rare sexual system among animals, called androdioecy, which is a coexistence of hermaphrodites and males in the population (Sawada *et al*., 2015). The males are small (dwarf males) and are attached to the hermaphrodites. Although being rare among animals, androdioecy is an intermediate condition linking combined and separate sexes, and therefore important to understand the evolution of sexual systems (Weeks, 2012; Pannell & Jordan, 2022).

In 2020, one of us (TY) found *O. unguisiformis* on a macrophthalmid *M. convexus*, not *M. milloti*, in Amami Oshima Island. This paper presents the new host record for *O. unguisiformis*.

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Materials and Methods

The macrophthalmid crab, harboring ectosymbiotic cirripedes, was found in Yanyu tidal flat in Kasari Bay, northern part of Amami Oshima Island (28.4159° N 129.5983° E) on 23 June 2020 (Fig. 1). The crab host and barnacles were preserved in 80% ethanol. The barnacle and the host crab were photographed using a digital camera (Olympus Tough TG-6). The morphological characters of barnacles were observed while referring to the original description of *Octolasmis unguisiformis* (Kobayashi & Kato 2003). The capitulum length of the barnacles was measured using a calibrated ocular micrometer under a dissection microscope. The hermaphrodite barnacles were dissected, and their egg masses, when found, were weighed by an analytical balance (A & D GR-200) to the nearest 0.1 mg. The crab was identified morphologically based on Komai *et al*. (1995). The breadth and length of the crab carapace were also measured. The crab sample (Specimen No. OMNH-Ar12881) and dwarf male barnacle sample (Specimen No. OMNH-Ar 12882.) were deposited in Osaka Museum of Natural History.

The molecular phylogenetic analysis was conducted using partial cytochrome c oxidase subunit I (COI) gene sequences. The total genomic DNA of the two barnacles and the crab was extracted from their soft body parts and preserved in 99% ethanol using Dneasy Blood & Tissue Kit (QIAGEN) following the manufacturer's instructions. The DNA sequence of the COI gene was multiplied using polymerase chain reaction (PCR). A fragment of the COI gene was amplified using the forward primer LCO-1490 and the reverse primer HCO-2198 (Folmer *et al*., 1994). The PCR amplification was conducted in 25 μL reaction volumes: 4.5 μL ddH_2O, 0.5 units of KOD FX Neo (TOYOBO), 12.5 μL PCR buffer for KOD FX Neo (2×), 5 μL dNTP mixture (each 2.0 mM), 0.75 μL of each primer (10 μM), and 1.0 μL genomic DNA. The thermal cycling was run under the following conditions: initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 98°C for 10 seconds, 30 seconds of annealing at 52°C, and extension for 60 seconds at 68°C. Amplicons were purified using ExoSAP-IT (Thermo Fisher Scientific). Sequencing reactions were prepared using ABI BigDye™ Terminator v3.1 Cycle Sequencing

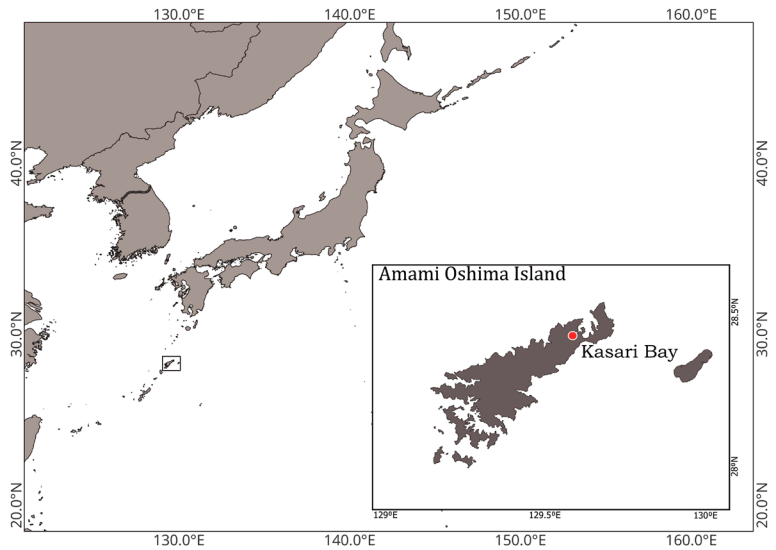

Fig. 1. Location of Kasari Bay, the northern part of Amami Oshima Island, Kagoshima Prefecture (red circle in the map).

Table 1. List of species and COI accession numbers in GenBank used for the genetic analyses.

Suborder	Family	Species	Accession no.	Locality	Reference
Lepadomorpha	Poecilasmatidae	*Octolasmis warwickii*	MH753552.1	Viet Nam	Unpublished
		Octolasmis warwickii	KC138501.1	Viet Nam-Ha Long Bay	Chen *et al*., 2013
		Octolasmis neptuni	MK541906.1	Viet Nam	Unpublished
		Octolasmis cor	KC138500.1	Taiwan	Chen *et al*., 2013
		Octolasmis cor	KC138499.1	Taiwan	Lin *et al*., 2015
		Octolasmis hawaiense	KF484230.1	Taiwan-The Philippines	Chan *et al*., 2016
		Octolasmis orthogonia	EU884173.1	Taiwan-The Philippines	Unpublished
		Octolasmis unguisiformis	LC467960.1	Japan-Kagoshima	Yamamori & Kato, 2020
		Octolasmis angulata	MN336860.1	Viet Nam-Quang Ninh	Unpublished
		Octolasmis unguisiformis	LC786870	Japan-Amami Oshima Island	This study
		Octolasmis unguisiformis	LC786871	Japan-Amami Oshima Island	This study
Pleocyemata	Macrophthalmidae	*Macrophthalmus japonicus*	LC097125.1	China-Qingdao	Teng & Shih, 2015
		Macrophthalmus japonicus	JX502913.1	South Korea	Unpublished
		Macrophthalmus banzai	LC097116.1	Taiwan-Gaomei Taichung	Teng & Shih, 2015
		Macrophthalmus pacificus	LC097131.1	China-Dongzhai Hainan	Teng & Shih, 2015
		Macrophthalmus pacificus	LC150454.1	China-Hainan	Teng & Shih, 2015
		Macrophthalmus milloti	LC097130.1	Japan-Ishigaki	Teng & Shih, 2015
		Macrophthalmus milloti	LC097129.1	Japan-Okinawa	Teng & Shih, 2015
		Macrophthalmus convexus	LC097118.1	Taiwan-Dongsha	Teng & Shih, 2015
		Macrophthalmus convexus	KC706708.1	French-Polynesia	Leray *et al*., 2013
		Macrophthalmus convexus	LC786872	Japan-Amami Oshima Island	This study

Kits (Applied Macrogen) following the manufacturer's protocol. The reaction mixture was analyzed by an ABI 3730xl Analyzer (Applied Macrogen). COI gene sequences were aligned using MEGA X (Tamura *et al*., 2018). Then, the BLAST (Basic Local Alignment Search Tool) analysis (at http://www.ncbi.nih.nlm.gov/) was carried out to determine the similarity of barnacle and crab samples to COI sequences in GenBank database (at http:// https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD = Web&PAGETYPE = BLASTHome) (Table 1). Phylogenetic trees were constructed using maximum likelihood (ML) analysis with MEGA X using the best models: the GTR + G model for the barnacle sequences and the HKY + I model for the crab sequences. Further, the robustness of the ML tree topology was assessed with 500 bootstrap replicates.

■ Results

Two hermaphrodite barnacles were found attached to the side walls of the carapace around the base of each cheliped and the first ambulatory leg of a female macrophthalmid crab (Fig. 2A, B). The capitulum lengths of the barnacles attached to the right side (Fig. 2C) and the left side (Fig. 2D) were 6.4 mm and 4.0 mm, respectively. The larger individual was ovigerous, and its egg mass was 9.2 mg. In addition, a dwarf male (1.19 mm capitulum length) was found attached to the larger individual (Fig. 2C). All the individuals had a branched scutum, three apparent projections in the tergum, and a curved, narrow and long carina. Based on the morphological traits of the shells, these individuals were identified as *Octolasmis unguisiformis* (Kobayashi & Kato, 2003). Moreover, a phylogenetic analysis based on the COI gene sequences of the two hermaphrodite individuals (Fig. 3) supported the morphological identification: they belong to a same clade with other *O. unguisiformis* sequences registered in the GenBank, with the bootstrap value of 100.

The host crab was an ovigerous female of 21.0 mm in carapace width and 12.7 mm in carapace length (Fig. 2). Based on morphology, this crab was identified as *Macrophthalmus* (*Macrophthalmus*) *convexus* Stimpson, 1858,

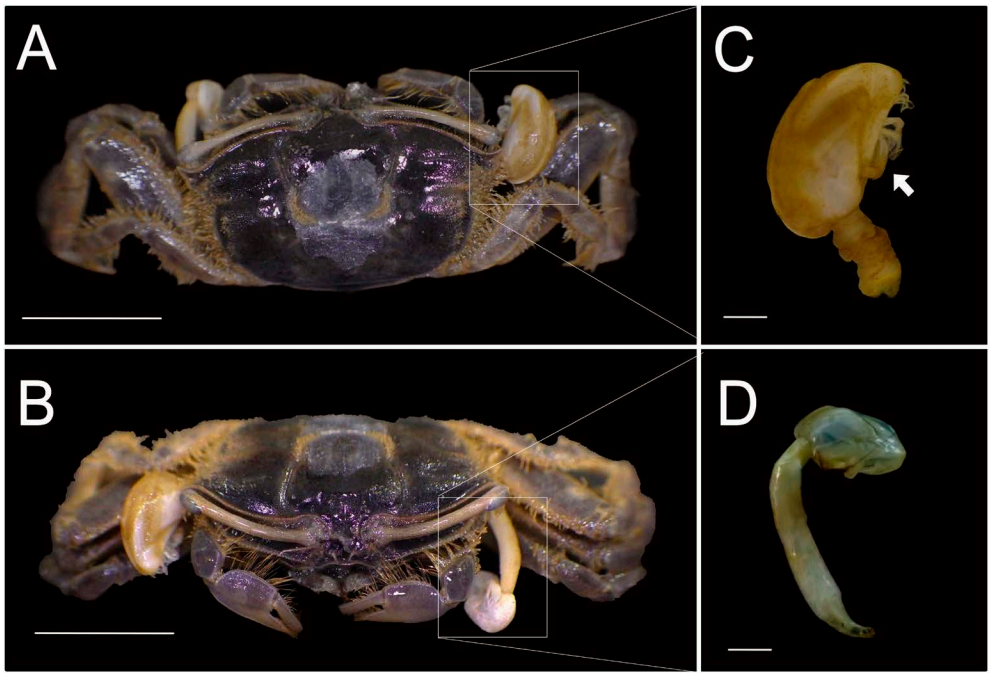

Fig. 2. (A, B) Two hermaphrodites of *Octolasmis unguisiformis*, attached to a crab, *Macrophthalmus convexus*. (C) Hermaphrodite on the right side, 6.4 mm in capitulum length, and a dwarf male (white arrow). (D) Hermaphrodite on the left side, 4.0 mm. Long bar = 10 mm, short bar = 1 mm.

as it has the following morphological characters: the carapace is broad and flattened, the lateral margin of the carapace has the external orbital tooth and two anterolateral teeth, the external orbital tooth projects considerably beyond the first anterolateral tooth, so the greatest carapace width is at the level of external orbital teeth. The ocular peduncle is slightly fell short of the tip of the external orbital tooth. Chelal fingers bear weak tuberculate teeth on the proximal half of the cutting margins, and the length of the movable finger is equal to that of the palm. Phylogenetic analysis based on the COI gene showed the crab specimen fits in the clade of *M. convexus* with a 100-bootstrap value (Fig. 4).

■ Discussion

Almost all octolasmid barnacles have broad host ranges (Jeffries *et al*., 1989; Negreiros-Fransozo *et al*., 1995; Jeffries & Voris, 1996, 2005). However, *Octolasmis unguisiformis* has been reported to occur only on *Macrophthalmus milloti* (Kobayashi & Kato, 2003; Sawada *et al*., 2015). This study has reported that two hermaphrodites and a dwarf male of *O. unguisiformis* were found attached to a closely-related species *M. convexus*. Thus, this study expands the host species record for *O. unguisiformis*, although it is still narrow compared with the host ranges of other octolasmids.

The host crabs of *O. unguisiformis*, i.e., *M. milloti* and *M. convexus*, have slightly different microhabitats at the littoral zones on the mud flats in the Ryukyu Archipelago; *M. convexus* prefers to live on relatively hard and sandy substrates at the lowest high intertidal zone, which is easily exposed to the air at low tides, whereas *M. milloti* occurs on soft bottoms of the intertidal zone of small estuaries (Komai *et*

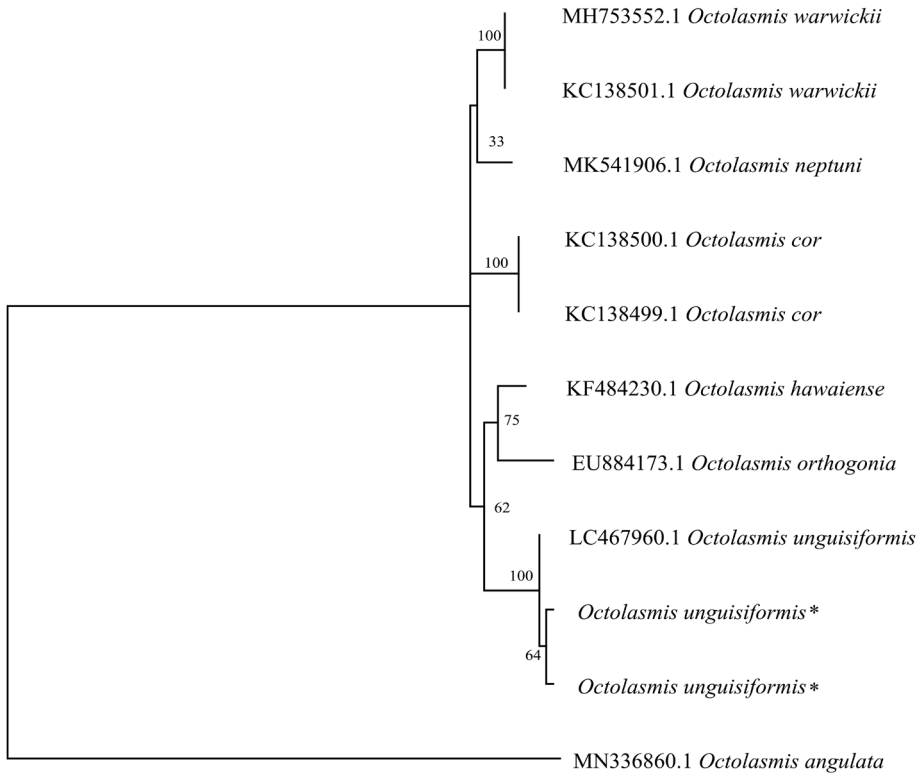

Fig. 3. ML phylogenetic tree based on COI gene sequences of octolasmid barnacles using the two individuals in this study (asterisks) and those from the published database. Numbers indicate bootstrap values and branch lengths numbers of substitutions per site.

al., 1995). Thus, the microhabitat of *M. convexus* may lead to desiccation and possible damage to *O. unguisiformis*. The attachment rate of *O. unguisiformis* was much lower for *M. convexus*; irrespective of our effort to collect more than 100 individuals of *M. convexus*, we could not find another case of *O. unguisiformis* on this crab (Wijayanti *et al.*, unpublished data), whereas the attachment rate is ca. 30% on *M. milloti* in Amami Oshima Island (Kobayashi & Kato, 2003).

The capitulum lengths of *O. unguisiformis* attached to *M. convexus* were large (6.4 and 4.0 mm), which is comparable to the largest individuals on *M. milloti* (7.1 mm in Kobayashi & Kato, 2003 and 6.6 mm in Sawada *et al*., 2015). The larger hermaphrodites of *O. unguisiformis* found in our study was ovigerous and carried a dwarf male, indicating that they could live at least a certain period and reproduce on *M. convexus*. The growth and survival of *Octolasmis* spp. are affected by the local environmental conditions, especially the ecdysis time of the host (Jeffries *et al*., 1989; Negreiros-Fransozo *et al*., 1995; Voris & Jeffries, 1997; Voris *et al*., 2000; Machado *et al*., 2013). However, there has been no data on the molting intervals for the two crab species.

In conclusion, the present study provides a new host record for the endangered barnacle *O. unguisiformis*. This information is important as it provides a clue for the adaptation and evolution of symbiotic organisms switching to different hosts. Moreover, this finding expands the possible microhabitat for *O. unguisiformis*. The growth and survival factors of this unique barnacle require further investigation.

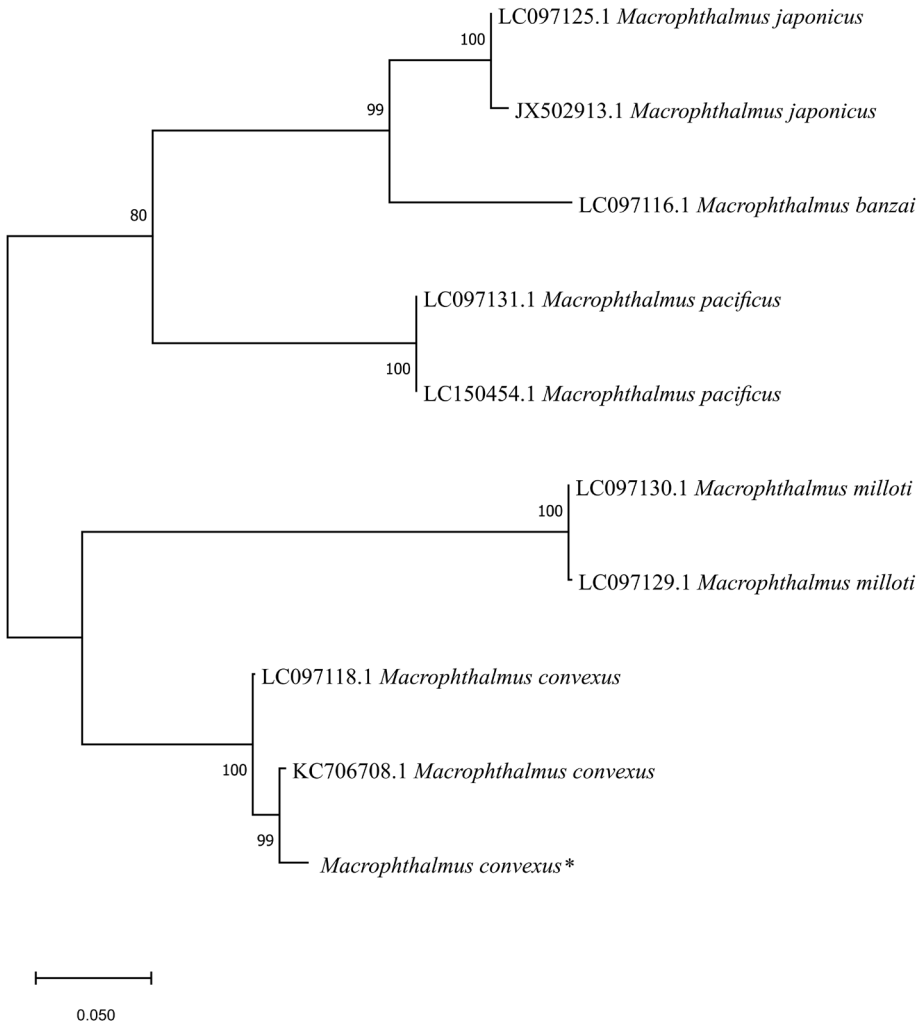

Fig. 4. ML phylogenetic tree based on COI gene sequences of macrophthalmid crabs using the individual in this study (asterisk) and those from the published database. Numbers indicate bootstrap values and branch lengths numbers of substitutions per site.

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